PCT/GB 2004 / 0 5 0 0 2 4

GB04/50024

INVESTOR IN PEOPLE

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

PRIORITY DOCUMENT

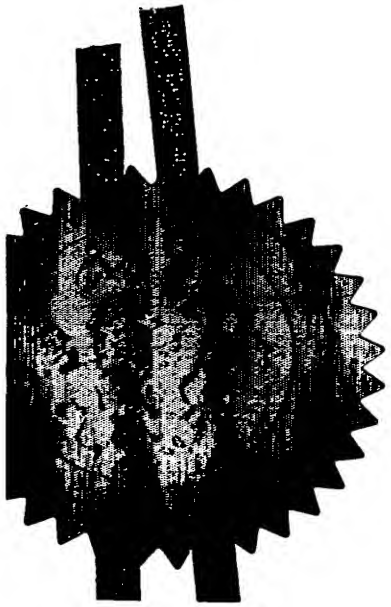
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.



Signed

Dated 16 November 2004

Patents Form 1/77

Patents Act 1977
(Rule 16)

The Patent Office

10NOV03 E850557-2 D10176
P01/7700 0.00-0326064.3

THE PATENT OFFICE
JR
- 7 NOV 2003
RECEIVED BY FAX

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference GBP290090

2. Patent application number (The Patent Office will fill in this part) 0326064.3

- 7 NOV 2003

3. Full name, address and postcode of the or of each applicant (underline all surnames)

Novexin Limited,
Babraham BioIncubator
Babraham
Cambridgeshire CB2 4AT
United Kingdom

8713281002

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation United Kingdom

4. Title of the invention Separation Methods

5. Name of your agent (if you have one)
"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

Marks & Clerk
66-68 Hills Road
Cambridge
CB2 1LA

Patents ADP number (if you know it) 18001

7/11/2003

6. Priority: Complete this section if you are declaring priority from one or more earlier patent applications, filed in the last 12 months

Country	Priority application No (if you know it)	Date of filing (day / month / year)

7. Divisionals, etc: Complete this section only if this application is a divisional application or resulted from an entitlement dispute

Number of earlier application	Date of filing (day / month / year)

8. Is a Patents Form 7/77 (Statement of inventorship and of right to grant of a patent) required in support of this request? Yes

(Answer 'Yes' if:
a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

Patents Form 1/77

9. Accompanying documents: A patent application must include a description of the invention. Not counting duplicates, please enter the number of pages of each item accompanying this form:

Continuation sheets of this form	0
Description	21
Claim(s)	7
Abstract	1
Drawing(s)	6

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent (Patents Form 7/77)	
Request for preliminary examination and search (Patents Form 9/77)	
Request for substantive examination (Patents Form 10/77)	
Any other documents (please specify)	

11. I/We request the grant of a patent on the basis of this application.

Signature(s) Marks & Clerk Date: 7 November 2003

12. Name and daytime telephone number of person to contact in the United Kingdom

Philip Martin
01223 345520

DUPLICATE

M&C Folio: GBP290090

Separation methods

The invention relates to separation methods for separation, purification and isolation of molecules, based on hydrophobic interaction chromatography and on mixed-mode hydrophobic interaction/ion exchange chromatography.

Background

Liquid chromatography is a commonly used protein purification technique due to its high capacity and selectivity. The technique depends upon the interactions of macromolecules in solution with a packed bed of a chromatography matrix. Interactions between the macromolecules and chromatography matrix may be based on size, charge, hydrophobicity or a more specific type of interaction (e.g. antibody – antigen binding). This leads to four broad classes of chromatography; size exclusion (gel permeation), ion exchange, hydrophobic interaction and affinity.

Ion exchange is the most commonly used method for the preparative scale purification of proteins, polypeptides, nucleic acids and other charged biomolecules (Bonnerjera et al., 1986, Bio/Technology; Freitag and Horvath, Advances in Biochemical Engineering/Biotechnology, 1995.). The advantages of ion exchange chromatography over alternative methods are its widespread

applicability, relative simplicity, high capacity and the relatively low cost of ion exchange matrices.

Separation of molecules during ion exchange chromatography depends on the reversible adsorption (non-covalent association) of charged molecules with a charged chromatography matrix. The molecule(s) of interest may carry the opposite charge to the chromatography matrix under the selected experimental conditions. The first stage in such purification is binding of the target molecule to the matrix through electrostatic interactions. This is followed by an elution step where the molecule is released from the matrix. Release can be achieved by altering the solution pH or ionic strength to create conditions that are unfavourable for molecule – matrix interactions, so that the association is disrupted and the molecule can be eluted from the chromatography matrix.

Elution is typically performed by introducing a gradually increasing salt concentration to the column. Weakly bound molecules are eluted first followed by more strongly bound substances. Such purifications can also be performed under conditions where the target molecule does not bind to the chromatography matrix, instead it passes through the matrix, the contaminants being removed through binding to the column.

Both anion exchange (positively charged) and cation exchange (negatively charged) matrices are commercially available. Such matrices can have varying degrees of ionisation, depending on pH. Strong ion exchangers are completely ionised over a wide pH range, whilst the ionisation and hence binding capacity,

of weak ion exchangers is more easily influenced by changes in buffer pH. Typical functional groups on the surface of ion exchange matrices include quarternary ammonium (strong anion), sulfonic acid (strong cation), diethylaminoethyl (weak anion) and carboxymethyl (weak cation).

Cyclodextrin and charged cyclodextrin derivatives have been used in capillary electrophoresis as selectivity and resolution enhancers. During electrophoresis, protein migration is driven by the application of an electric field. Many types of detergents and certain types of cyclodextrins have been used to alter the behaviour of various proteins during electrophoresis (Weinberger, Practical Capillary Electrophoresis, 2000; Rathore and Horvath, Electrophoresis, 1998). Covalently immobilised cyclodextrin has been used as chiral chromatography matrices for enantiomer separation, these separations are based on the stereoselectivity for the cyclodextrin cavity and do not involve a charged stationary phase such as an ion exchange chromatography matrix.

Disclosure of the Invention

The present invention provides a method for chromatographic separation of a molecule, wherein a charged amphipathic sugar polymer(s) is employed to modify the hydrophobic interaction between the molecule and a charged stationary phase.

In methods of the invention, charged amphipathic sugar polymers alter the level of interaction between the molecules, in particular charged molecules and a

charged stationary phase such as an ion exchange resin. The separation methods of the invention are useful for separation, purification and isolation of molecules based on hydrophobic interaction chromatography or on mixed-mode hydrophobic interaction/ion exchange chromatography.

The present invention provides a method for separating a molecule from a solution comprising the molecule and further components by hydrophobic interaction chromatography comprising applying the solution comprising the molecule to a charged stationary phase, and eluting the molecule from the stationary phase in a mobile phase, characterised in that:

(a) the charged stationary phase is non-covalently associated with a charged amphipathic sugar polymer(s), or the stationary phase comprises a charged amphipathic sugar polymer, and/or

(b) the molecule is non-covalently associated with a charged amphipathic sugar polymer(s).

In some methods of the invention, the pH chosen for the solution comprising the molecule and/or for the mobile phase is below the pI of the molecule and thus the molecule carries a net positive charge. Alternatively, the pH of the solution and/or the mobile phase can be chosen so that it is above the pI of the molecule and thus the molecule carries a net negative charge. The solution comprising the molecule may be prepared by dissolving the molecule in the mobile phase running buffer or by diluting the molecule in the mobile phase running buffer.

The present invention provides a method for separating a positively charged molecule from a solution comprising the molecule and further components by hydrophobic interaction chromatography comprising applying a solution comprising the molecule to a positively charged stationary phase (e.g. an anion exchanger) which is non-covalently associated with a negatively charged amphipathic sugar polymer(s) (e.g. sulfated beta-cyclodextrin), and eluting the molecule from the stationary phase in a mobile phase.

The present invention further provides a method for separating a negatively charged molecule from a solution comprising the molecule and further components by hydrophobic interaction chromatography comprising applying a solution comprising the molecule to a negatively charged stationary phase (e.g. a cation exchanger) which is non-covalently associated with a positively charged amphipathic sugar polymer(s), and eluting the molecule from the stationary phase in a mobile phase.

The present invention also provides a method for separating a positively charged molecule from a solution comprising the molecule and further components by mixed mode hydrophobic interaction/ion exchange chromatography comprising applying a solution comprising the molecule to a negatively charged stationary phase (e.g. a cation exchanger) which is non-covalently associated with a positively charged amphipathic sugar polymer(s) (e.g. amino beta-cyclodextrin), and eluting the molecule from the stationary phase in a mobile phase.

The present invention further provides a method for separating a negatively charged molecule from a solution comprising the molecule and further components by mixed mode hydrophobic interaction/ion exchange chromatography comprising applying a solution comprising the molecule to a positively charged stationary phase (e.g. an anion exchanger) which is non-covalently associated with a negatively charged amphipathic sugar polymer(s) (e.g. a carboxymethyl cyclodextrin), and eluting the molecule from the stationary phase in a mobile phase.

The mobile phase employed in methods of the invention may comprise an amphipathic sugar polymer(s), i.e. an amphipathic sugar polymer or a mixture of amphipathic sugar polymers. The mobile phase may comprise a charged amphipathic polymer(s). In methods where the amphipathic sugar polymer(s) are included in the solution and/or mobile phase (e.g. running buffer) the amphipathic sugar polymer or mixture of amphipathic sugar polymers is suitably present at from 0.01 – 50 mg/ml, preferably from 0.01 to 20 mg/ml, more preferably from 0.1 to – 10mg/ml.

The invention provides a method for separating a positively charged molecule from a solution comprising the molecule and further components by mixed mode hydrophobic interaction/ion exchange chromatography comprising applying a solution comprising the molecule to a negatively charged stationary phase (e.g. a cation exchanger) to the stationary phase, and eluting the molecule from the stationary phase in a mobile phase comprising a negatively charged amphipathic polymer(s) (e.g. a carboxy methyl cyclodextrin).

The invention further provides a method for separating a negatively charged molecule from a solution comprising the molecule and further components by mixed mode hydrophobic interaction/ion exchange chromatography comprising applying a solution comprising the molecule to a positively charged stationary phase (e.g. an anion exchanger), and eluting the molecule from the stationary phase in a mobile phase comprising a positively charged amphipathic sugar polymer(s).

In an aspect of a method of the invention, the charged stationary phase may comprise or consist of a charged amphipathic sugar polymer(s).

Thus the invention provides a method for separating a molecule from a solution comprising a molecule and further components by hydrophobic interaction chromatography comprising applying the solution comprising the molecule to a charged stationary phase, and eluting the molecule from the stationary phase in a mobile phase, characterised in that the stationary phase comprises a charged amphipathic sugar polymer(s).

Also provided is a method for separating a molecule from a solution comprising a charged molecule and further components by hydrophobic interaction chromatography comprising applying the solution comprising the molecule to an oppositely charged stationary phase, and eluting the molecule from the stationary phase in a mobile phase, characterised in that the stationary phase comprises a charged amphipathic sugar polymer(s).

A charged stationary phase that comprises a charged amphipathic sugar polymer(s) may take the form of a charged amphipathic sugar polymer(s) immobilised within a support (e.g. a polyacrylamide gel crosslinked with cyclodextrin) or immobilised on a support. Suitable supports include agarose, acrylate, cellulose, dextrin, polystyrene, polyacrylamide, Sepharose and silica. The invention further provides a charged amphipathic sugar polymer immobilised on a support, e.g. agarose, acrylate, cellulose, dextrin, polystyrene, polyacrylamide, Sepharose, silica. Alternatively, the charged stationary phase may consist of one or a mixture of charged amphipathic sugar polymer(s).

The charged stationary phase may be generated by temporary derivatisation of an ion exchange matrix, suitably by contacting a solution comprising the amphipathic sugar polymer(s) with the ion exchange matrix, e.g. by running a solution comprising the amphipathic sugar polymer(s) through an ion exchange column. Suitable concentrations of amphipathic sugar polymers for derivatisation of ion exchange matrices are from 0.01 to 500 mg sugar polymer/ml matrix, preferably from 0.1 to 50 mg sugar polymer/ml matrix, most preferably from 1 to 30 mg sugar polymer/ml matrix.

The sugar polymers used in methods of the invention must have an amphipathic nature (both hydrophilic and hydrophobic surfaces).

A mixture of amphipathic sugar polymers may be a mixture of positively charged amphipathic sugar polymers, or a mixture of negatively charged amphipathic sugar polymers. Alternatively the mixture of amphipathic sugar

polymers can be a mixture of positively and negatively charged sugar polymers which mixture itself may be positively or negatively charged depending on the relative proportions and charge of the charged species.

An amphipathic sugar polymer useful in a method of the invention can be a cyclic sugar polymer, preferably a glucosan or a derivative thereof, more preferably a cyclodextrin or derivative thereof, most preferably an α-cyclodextrin, a β-cyclodextrin, a γ-cyclodextrin, or a derivative thereof. β-cyclodextrin and derivatives thereof are particularly favoured. Suitable cyclodextrin derivatives for use in methods of the invention include negatively charged derivatives such as cyclodextrin sulfate, sulfopropyl cyclodextrin, sulfobutylether cyclodextrin, cyclodextrin phosphate, carboxymethyl cyclodextrin, carboxyethyl cyclodextrin and succinyl hydroxypropyl cyclodextrin; and positively charged derivatives such as quarternary ammonium cyclodextrin and 6-monodeoxy-6-monoamino cyclodextrin.

An amphipathic sugar polymer useful in a method of the invention can be a helical sugar polymer, preferably as a fructosan or a derivative thereof, more preferably an inulin or a derivative thereof.

The term amphipathic sugar polymer(s) encompasses a single type of amphipathic sugar polymer or a mixture of amphipathic sugar polymers.

In some instances, the eluate collected after separation containing the separated molecule may contain amphipathic polymer(s). If desired, the

amphipathic polymer(s) can be removed from the eluate, and thus from the molecule. Methods for removal of amphipathic polymer(s) from the eluate include dialysis, diafiltration, and/or size exclusion chromatography. The amphipathic sugar polymer(s) can be removed by degradation of the amphipathic sugar polymer(s), which can be performed by one or more of the following methods: chemical degradation, enzymic digestion, electromagnetic radiation, shear stress, or heat. Preferably degradation is by enzymic digestion, more preferably degradation is by glucosyltransferase, amylase and/or xylanase digestion. Enzymic degradation can be performed by contacting the eluate containing amphipathic polymer(s) with immobilised enzyme, for example by passing the eluate through a column containing immobilised enzyme. The sugar polymer can be degraded to its constituent monomers, the molecule of interest can then be purified using a method such as dialysis, diafiltration, and/or size exclusion chromatography.

Separation methods of the invention can be used to separate, isolate and purify molecules that exhibit some degree of surface exposed hydrophobicity, i.e. molecules that are hydrophobic or amphiphilic. The methods are particularly useful to separate protein molecules, preferably proteins with one or more hydrophobic side chains, (e.g. a protein containing tryptophan). The term protein as used herein encompasses proteins, peptides, polypeptides and oligopeptides, enzymes, monomeric proteins and multimeric proteins which can be homomers or heteromers. Proteins may be synthetic or naturally occurring, and may be obtained by chemical synthesis, or by recombinant or non-recombinant methods. Separation methods of the invention are also particularly

useful for separation, isolation and purification of a nucleic acid molecule, such as single or double stranded DNA or RNA or a DNA/RNA heteroduplex.

In a preferred separation method of the invention, the molecule of interest is a charged protein and is separated from further protein(s) present in the solution. The method is particularly useful for separation of a charged protein from further proteins in solution, especially from further similarly charged protein(s). In particular, purification of two proteins with differing hydrophobicities but very similar charge can be achieved using methods of the invention; such separations are currently extremely difficult or impossible to perform using existing ion exchange chromatography methods. Separations can sometimes be achieved using electrophoretic methods, but these generally require that the mobile phase be at an extreme pH, e.g. a very acidic pH in the range pH2 to pH 5. These aggressive low pH conditions may cause degradation or otherwise affect the molecule of interest. If the molecule of interest is a protein, it is liable to be denatured at low pH and inactivated. Thus after separation by electrophoretic methods it may be necessary to attempt to renature the protein, which may not be successful and will result in loss of yield of native, functional protein. Methods of the invention are advantageous as they can be used to achieve effective separation of molecules, in particular molecules such as proteins and nucleic acids, at pH in the range 5 to 9, so it is possible to perform the separation methods using reaction conditions that do not result in significant degradation of, or damage to, the molecule of interest.

Where a protein or proteins are to be separated using a method of the invention the interaction between amphipathic sugar polymers and hydrophobic amino

acid side chains of the protein(s) of interest can be modulated. The amphipathic sugar polymer – protein interaction can be used to increase or decrease the affinity of the protein for the chromatography matrix. Under such conditions the protein – matrix interaction will be determined by the hydrophobic protein – sugar polymer interactions, in addition to or instead of the charge based protein – matrix interactions. In free solution, binding of the charged amphipathic sugar polymer to the protein changes the effective net charge of the protein, altering its behaviour during ion exchange purification. Binding of the charged amphipathic sugar polymer to the charged stationary phase (ion exchange chromatography matrix) allows the matrix to interact with the protein through charge and/or hydrophobic effects. Therefore, methods of the invention can solve the problem of separating similarly charged proteins using ion exchange chromatography.

List of figures

In each of the figures conductivity (mS/cm) and UV absorbance at 280nm (mAu) of eluted fractions are plotted against time (min).

Figure 1 shows the results when acetone in aqueous solution run on a non-derivatised Q-Sepharose anion exchange column. Acetone is not retained on the column.

Figure 2 shows the results when lysozyme in aqueous solution is run on a non-derivatised Q-Sepharose anion exchange column. Lysozyme is positively charged and is not retained on the positively charged column.

Figure 3 shows the results when a mixture of acetone and lysozyme in aqueous solution is run on a non-derivatised Q-Sepharose anion exchange column. Neither the acetone, nor the lysozyme is retained on the column.

Figure 4 shows the results when acetone in aqueous solution is run on a Q-Sepharose anion exchange column derivatised with sulfated beta cyclodextrin. Acetone is not retained on the derivatised column.

Figure 5 shows the results when lysozyme in aqueous solution is run on a Q-Sepharose anion exchange column derivatised with sulfated beta cyclodextrin. Lysozyme is retained on the derivatised column and is eluted from the column (peak at 13 to 20 minutes) using a salt gradient.

Figure 6 shows the results when a mixture of acetone and lysozyme in aqueous solution is run on a Q-Sepharose anion exchange column derivatised with sulfated beta cyclodextrin. Acetone is not retained on the column and is eluted from the column in under five minutes. Lysozyme is retained on the derivatised column and is eluted from the column (peak at 13 to 20 minutes) using a salt gradient. Using the derivatised column two UV absorbance peaks are seen confirming that the acetone and lysozyme have been separated on the derivatised column.

Examples

The following methods are for the purification of basic proteins (high pI). Resin types (charge) would be reversed to separate acidic proteins.

Example 1 - Method 1 separation of a strongly hydrophobic protein:

A running buffer (mobile phase) is selected with a pH below the pI of the protein so that the protein carries a positive charge. Purification of the protein is achieved using a negatively charged sugar polymer carboxymethyl beta-cyclodextrin and a positively charged chromatography matrix (anion exchanger). Traditionally it would not be possible to retain a positively charged protein on a positively charged matrix. However, in this method, a solution of a negatively charged sugar polymer is injected through the column prior to introduction of the protein solution. The sugar polymer binds to the chromatography matrix, temporarily derivatising the surface. This derivatised surface is capable of hydrophobic interaction with protein molecules. The protein(s) to be separated are then injected through the column. An elution is performed. The elution method can be an isocratic elution (no salt gradient) in which retention time on the column is determined by the strength of the hydrophobic protein – sugar polymer interactions. A protein separation is achieved, the most hydrophobic protein is eluted last from the column. Thus a separation based on protein hydrophobicity is achieved using an ion-exchange matrix. Alternatively a salt gradient may be used for the elution. The elution may also involve the use of one or more other compounds (e.g. urea, methanol,

ethanol, isopropyl alcohol, guanidine hydrochloride or acetonitrile) to modulate the hydrophobic interaction of the sugar polymer with protein.

Chromatography column:

Type	Strong anion exchanger
Brand	Q sepharose fast flow
Manufacturer	Amersham
Size	1ml

Running buffers:

Tris	100mM
EDTA	1mM
pH	8.0
Flow	1ml/min
Salt gradient	NaCl 0-0.5M

Target protein:

Type	Lysozyme (pI=11.3)
Concentration	0.5mg/ml
Inject volume	1ml

Sugar polymer:

Description	Negatively charged, cyclodextrin derivative
Type	Sulfated-beta-cyclodextrin
Conc	4mg/ml

Inject 25ml

A 1 ml Q Sepharose anion exchanger was employed to separate the protein lysozyme from acetone in aqueous solution. The process was operated as described in method 1 using a salt gradient to elute the protein and sugar polymer.

Lysozyme has a molecular weight of 14 kDa and an isoelectric point of 11.3. The running buffer used was 100 mM Tris, 1 mM EDTA at pH 8.0 (buffer 1). Therefore, both the protein and the chromatography stationary phase were positively charged under the process conditions. Charged molecules bound to the anion exchange resin were removed by elution using a salt gradient. This was performed by mixing buffer 1 with buffer 2 (as buffer 1 plus 1 M NaCl) in gradually increasing ratios of buffer 2.

The column was loaded with 1 ml samples for each of the six runs shown. The samples contained 0.25% v/v acetone, 0.5 mg/ml lysozyme or a mixture of 0.25% v/v acetone, 0.5 mg/ml lysozyme. All samples were dissolved in buffer 1. Each sample was run on the Q Sepharose column with a flow rate of 1ml/min. The experiments were performed by first running each of the acetone, lysozyme and mixed acetone/lysozyme samples individually down the underivatised column. This process was repeated using the same column, but using a derivatisation step to derivatise the column with sulfated-beta-cyclodextrin before running each sample down the column. To derivatise the column the sulphated beta-cyclodextrin was loaded onto the column by injecting

25ml of buffer 1 containing 4 mg/ml of the sulphated beta-cyclodextrin at a flow rate of 4 ml/min. The sugar polymer used, sulphated beta-cyclodextrin sodium salt (Aldrich, #38,915-3), is negatively charged.

Elution of acetone and lysozyme was monitored continuously by detecting UV absorbance at 280 nm (mAu) of elute. Lysozyme and acetone absorb UV light at 280 nm. Sulphated beta-cyclodextrin does not absorb UV light at 280 nm sufficiently strongly to affect the UV signals measured. Conductivity was measured in mS/cm. The conductivity of buffer 1 was around 8 mS/cm and of buffer 2 was around 80 mS/cm.

Under normal operation, (i.e. without derivatisation of the anion exchange resin), lysozyme cannot be retained on an anion exchange column as both the column and protein have the same charge. Since the flow rate is 1ml/min and the column volume is 1ml with around 50% voidage, material that cannot bind to the chromatography stationary phase begins to elute approximately 0.5 min after injection (figure 2).

Table 1

Sample	Sepharose anion exchange column - Non-derivatised	Sepharose anion exchange column - Derivatised with sulphated beta-cyclodextrin
Acetone	No retention (Fig 1)	No retention (Fig 4)
Lysozyme	No retention (Fig 2)	Retained (Fig 5)
Mixture of Acetone and Lysozyme	No retention (Fig 3)	Separated (Fig 6)

All three samples run on the underivatised column eluted rapidly without binding to the column (figures 1 to 3). The same process occurred when acetone was injected onto the sulphated beta-cyclodextrin-derivatised column (figure 4). Acetone is uncharged and thus does not interact with the anion exchange resin; acetone forms no hydrophobic interaction with cyclodextrin sugar polymer and so elutes without binding to either the non-derivatised or derivatised column.

When the lysozyme sample was injected onto the derivatised column, no increase in UV absorbance was detected in the 0 to 5 minute range, as the protein was retained on the derivatised column (figure 5). When a salt gradient was run down the column, the UV absorbance increased rapidly, indicating that the retained protein was being eluted from the column (figure 5) .

In the final run, a sample containing a mixture of acetone and lysozyme was found to be separated efficiently on the derivatised column. The acetone passed through the column, but the lysozyme protein was retained until it was eluted from the column using a salt gradient. These results demonstrate that hydrophobic sugar polymer – protein interactions can be used in ion-exchange chromatography to achieve separation.

Method 2 - Separation of a weakly hydrophobic protein

In this method a positively charged amphipathic sugar polymer (e.g. amino beta-cyclodextrin) and a negatively charged chromatography matrix (cation exchanger) are used. The column is derivatised with a large pulse of the

positively charged sugar polymer in aqueous solution. A solution containing the positively charged weakly hydrophobic protein is then injected through the column. This method is mixed mode, that is protein – matrix interactions occur through a combination of electrostatic and hydrophobic interactions. Elution is performed using a gradually increasing salt concentration to screen out the protein – matrix electrostatic interactions. However, since the protein is bound through a combination of electrostatic and hydrophobic interactions elution of the more hydrophobic protein will occur at a higher salt concentration. This method is particularly useful for separation of two proteins with very similar charge using an ion exchange matrix.

Chromatography column:

Type	Weak cation exchanger
Brand	CM sepharose fast flow
Manufacturer	Amersham
Size	1ml

Running buffers:

Tris	100mM
EDTA	1mM
pH	8.0
Flow	1ml/min
Salt gradient	NaCl 0-0.5M

Protein:

Type	Lysozyme (pI=11.3) with Cytochrome c (pI=10.6), alpha chymotrypsinogen (pI=9.5) or ribonuclease A (pI=9.6)
Concentration	0.5mg/ml
Inject volume	1ml

Sugar polymer:

Description	Weakly positively charged, cyclodextrin derivative
Type	6-monodeoxy-6-monoamino-beta-cyclodextrin hydrochloride
Conc	4mg/ml
Inject	25ml

Method 3

In this method, the hydrophobic interaction between the amphipathic sugar polymer and the positively charged protein of interest is used to decrease binding to the chromatography matrix. The sugar polymer used has a weak negative charge (e.g. carboxymethyl beta-cyclodextrin). A negatively charged chromatography matrix (cation exchanger) is used to ensure that there is little or no binding of the sugar polymer to the matrix. The positively charged protein is passed through the column and binds to the negatively charged matrix. The sugar polymer is now included in the elution buffer and a salt gradient passed through the column. The sugar polymer binds to the most hydrophobic protein with greatest affinity. This reduces the protein net charge and allows the most

hydrophobic protein to be eluted at a lower salt concentration than a similarly charged less hydrophobic protein.

Method 4 - Optional Step – Removal of amphipathic sugar polymer(s)

Eluent obtained from the ion exchange column is passed through a second column, or column section, containing an immobilised enzyme. The enzyme degrades any sugar polymer that co-elutes with the protein(s) of interest. Sugar polymers are degraded to monomers, e.g. glucose, fructose and/or derivatives thereof. This releases the sugar polymers from the protein molecules and prevents any further modification of the protein properties by the sugar polymer.

CLAIMS:

1. A method for chromatographic separation of a molecule, wherein a charged amphipathic sugar polymer(s) is employed to modify the hydrophobic interaction between the molecule and a charged stationary phase.

2. A method for separating a molecule from a solution comprising the molecule and further components by hydrophobic interaction chromatography comprising applying the solution comprising the molecule to a charged stationary phase, and eluting the molecule from the stationary phase in a mobile phase; characterised in that:

(a) the charged stationary phase is non-covalently associated with a charged amphipathic sugar polymer(s), or the stationary phase comprises a charged amphipathic sugar polymer, and/or

(b) the molecule is non-covalently associated with a charged amphipathic sugar polymer(s).

3. A method according to claim 1 or claim 2 wherein the pH of the mobile phase is below the pI of the molecule (and thus the molecule carries a net positive charge).

4. A method according to claim 1 or claim 2 wherein the pH of the mobile phase is above the pI of the molecule (and thus the molecule carries a net negative charge).

5. A method according to claim 3 for separating a positively charged molecule from a solution comprising the molecule and further components by hydrophobic interaction chromatography comprising applying a solution comprising the molecule to a positively charged stationary phase which is non-covalently associated with a negatively charged amphipathic sugar polymer(s), and eluting the molecule from the stationary phase in a mobile phase.

6. A method according to claim 4 for separating a negatively charged molecule from a solution comprising the molecule and further components by hydrophobic interaction chromatography comprising applying a solution comprising the molecule to a negatively charged stationary phase which is non-covalently associated with a positively charged amphipathic sugar polymer(s), and eluting the molecule from the stationary phase in a mobile phase.

7. A method according to claim 3 for separating a positively charged molecule from a solution comprising the molecule and further components by mixed mode hydrophobic interaction/ion exchange chromatography comprising applying a solution comprising the molecule to a negatively charged stationary phase which is non-covalently associated with a positively charged amphipathic sugar polymer(s), and eluting the molecule from the stationary phase in a mobile phase.

8. A method according to claim 4 for separating a negatively charged molecule from a solution comprising the molecule and further components by mixed mode hydrophobic interaction/ion exchange chromatography comprising

applying a solution comprising the molecule to a positively charged stationary phase which is non-covalently associated with a negatively charged amphipathic sugar polymer(s), and eluting the molecule from the stationary phase in a mobile phase.

9. A method according to any of the preceding claims wherein the mobile phase comprises an amphipathic sugar polymer(s).

10. A method according to any of the preceding claims wherein the mobile phase comprises a charged amphipathic sugar polymer(s).

11. A method according to claim 7 for separating a positively charged molecule from a solution comprising the molecule and further components by mixed mode hydrophobic interaction/ion exchange chromatography comprising applying a solution comprising the molecule to a negatively charged stationary phase, and eluting the molecule from the stationary phase in a mobile phase comprising a negatively charged amphipathic sugar polymer(s).

12. A method according to claim 8 for separating a negatively charged molecule from a solution comprising the molecule and further components by mixed mode hydrophobic interaction/ion exchange chromatography comprising applying a solution comprising the molecule to a positively charged stationary phase, and eluting the molecule from the stationary phase in a mobile phase comprising a positively charged amphipathic sugar polymer.

13. A method according to any preceding claim wherein the or an amphipathic sugar polymer is a cyclic sugar polymer.

14. A method according to claim 13 wherein the or an amphipathic sugar polymer is a glucosan or a derivative thereof.

15. A method according to claim 14 wherein the or an amphipathic sugar polymer is a cyclodextrin or derivative thereof.

16. A method according to claim 15 wherein the cyclodextrin is an α-cyclodextrin, a β-cyclodextrin, a γ-cyclodextrin, or a derivative thereof.

17. A method according to claim 16 wherein the cyclodextrin is a β-cyclodextrin derivative.

18. A method according to any one of claims 15 to 17 wherein the cyclodextrin derivative(s) is selected from the group comprising cyclodextrin sulfate, sulfopropyl cyclodextrin, sulfobutylether cyclodextrin, cyclodextrin phosphate, carboxymethyl cyclodextrin, carboxyethyl cyclodextrin, succinyl hydroxypropyl cyclodextrin, quarternary ammonium cyclodextrin and 6-monodeoxy-6-monoamino cyclodextrin.

19. A method according to any of claims 1 to 12 wherein the amphipathic sugar polymer is a helical sugar polymer.

20. A method according to claim 19 wherein the amphipathic sugar polymer is a fructosan or a derivative thereof.

21. A method according to claim 20 wherein the amphipathic sugar polymer is an inulin or a derivative thereof.

22. A method according to any preceding claim further comprising removal of amphipathic sugar polymer(s) from the eluate.

23. A method according to claim 22 wherein removal of amphipathic sugar polymer(s) from the eluate is by degradation of amphipathic sugar polymer(s) in the eluate.

24. A method according to claim 23 wherein degradation of the amphipathic sugar polymer(s) is by one or more of the following methods: chemical degradation, enzymic digestion, electromagnetic radiation, shear stress or heat.

25. A method according to claim 23 or 24 wherein degradation is in part or fully by enzymic digestion.

26. A method according to claim 25 wherein enzymic digestion is by glucosyltransferase, amylase and/or xylanase digestion.

27. A method according to claims 25 or 26 wherein enzymic digestion is by contacting the eluate with immobilised enzyme.

28. A method according to claim 27 wherein contacting is by passing eluate through a column containing immobilised enzyme.

29. A method according to any preceding claim wherein the molecule for separation is hydrophobic or amphipathic.

30. A method according to any preceding claim wherein the molecule for separation is a protein or a nucleic acid.

31. A method according to any preceding claim wherein the molecule for separation is a charged protein and is separated from further protein(s).

32. A method according to any preceding claim wherein the molecule for separation is a charged protein and is separated from further similarly charged protein(s).

33. A method according to any preceding claim characterised in that the charged stationary phase comprises a charged amphipathic sugar polymer(s).

34. A method for separating a molecule from a solution comprising a molecule and further components by hydrophobic interaction chromatography comprising applying the solution comprising the molecule to a charged stationary phase and eluting the molecule from the stationary phase in a mobile phase characterised in that the stationary phase comprises a charged amphipathic sugar polymer(s).

35. A method for separating a molecule from a solution comprising a charged molecule and further components by hydrophobic interaction chromatography comprising applying the solution comprising the molecule to an oppositely charged stationary phase, and eluting the molecule from the stationary phase in a mobile phase, characterised in that the stationary phase comprises a charged amphipathic sugar polymer(s).

ABSTRACT:

A method for chromatographic separation of a molecule, wherein a charged amphipathic sugar polymer(s) is employed to modify the hydrophobic interaction between the molecule and a charged stationary phase.

Figure 1

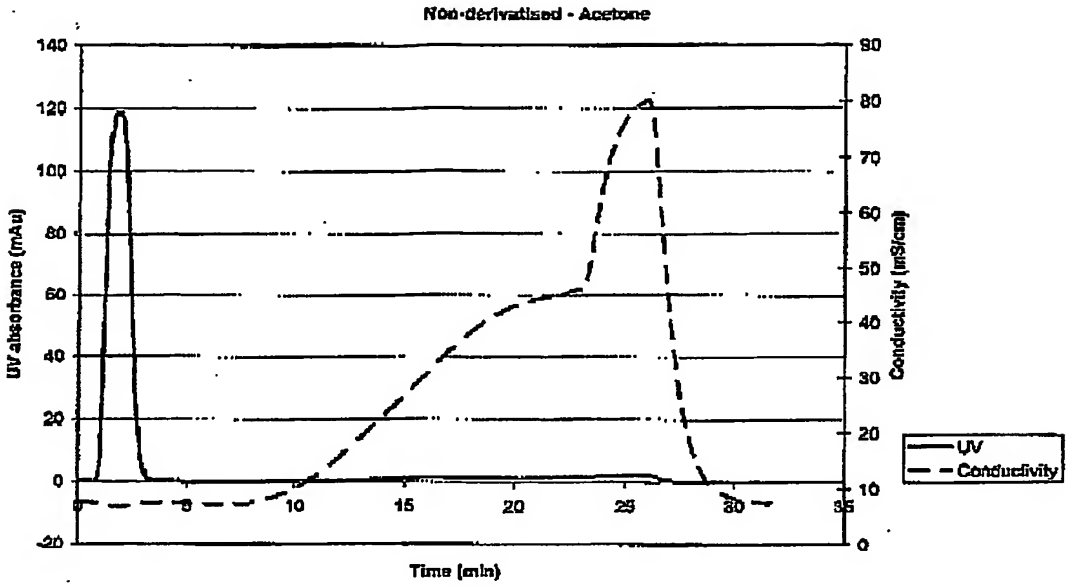

Non-derivatised - Acetone
UV absorbance (mAu)
Conductivity (mS/cm)
Time (min)
UV
Conductivity

Figure 2

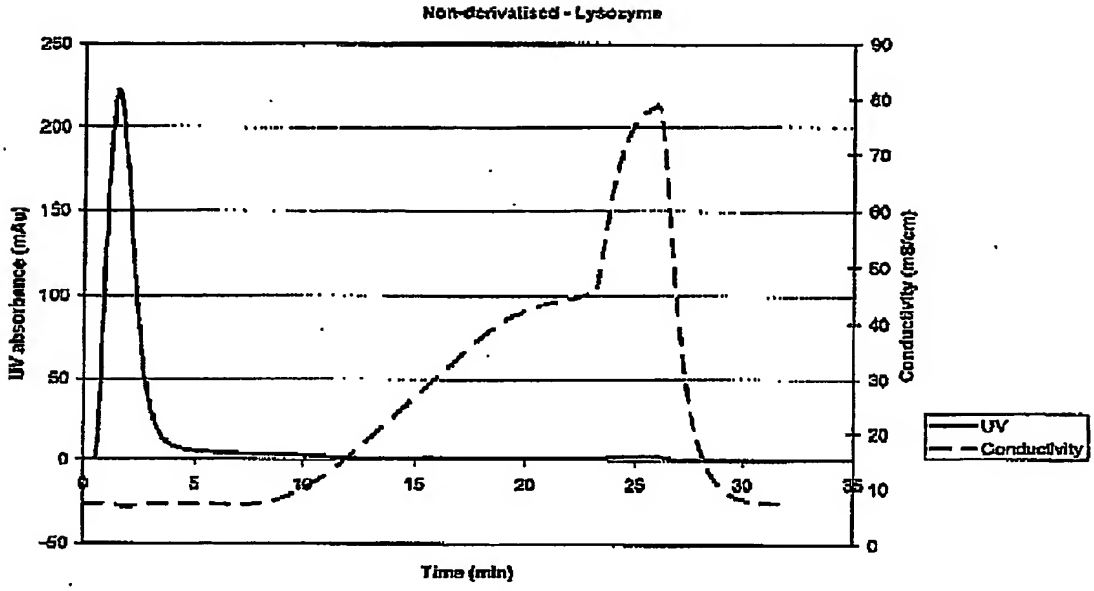

Non-derivatised - Lysozyme
UV absorbance (mAu)
Conductivity (mS/cm)
Time (min)
UV
Conductivity
250
200
150
100
50
0
-50
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35

Figure 3

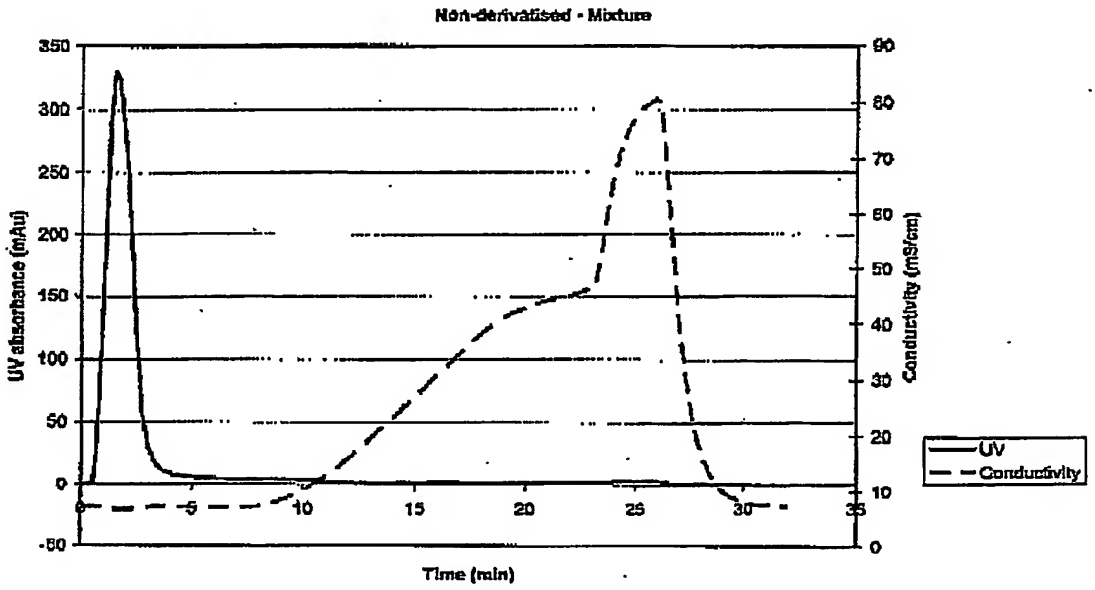

Non-derivatised - Mixture
UV absorbance (mAu)
Conductivity (mS/cm)
Time (min)
UV
Conductivity
350
300
250
200
150
100
50
0
-50
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35

Figure 4

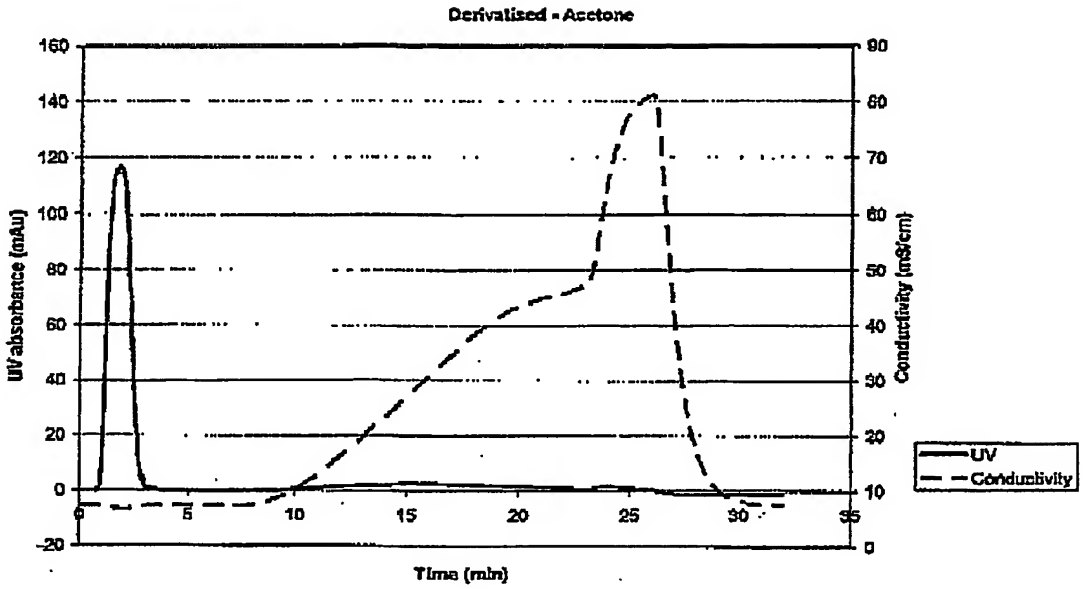

Derivatised - Acetone
UV absorbance (mAu)
Conductivity (mS/cm)
Time (min)
UV
Conductivity
160
140
120
100
80
60
40
20
0
-20
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35

Figure 5

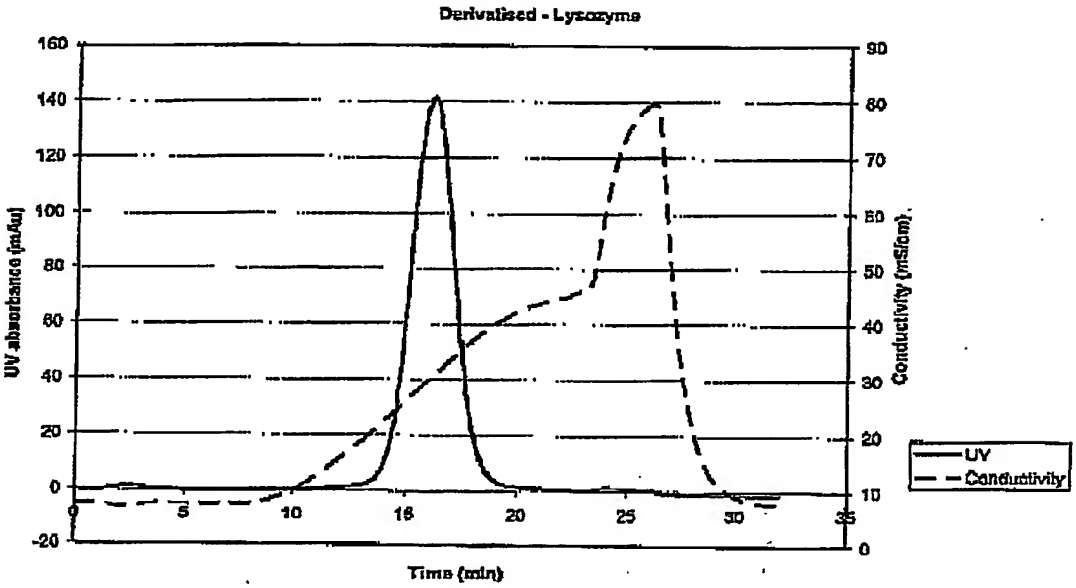
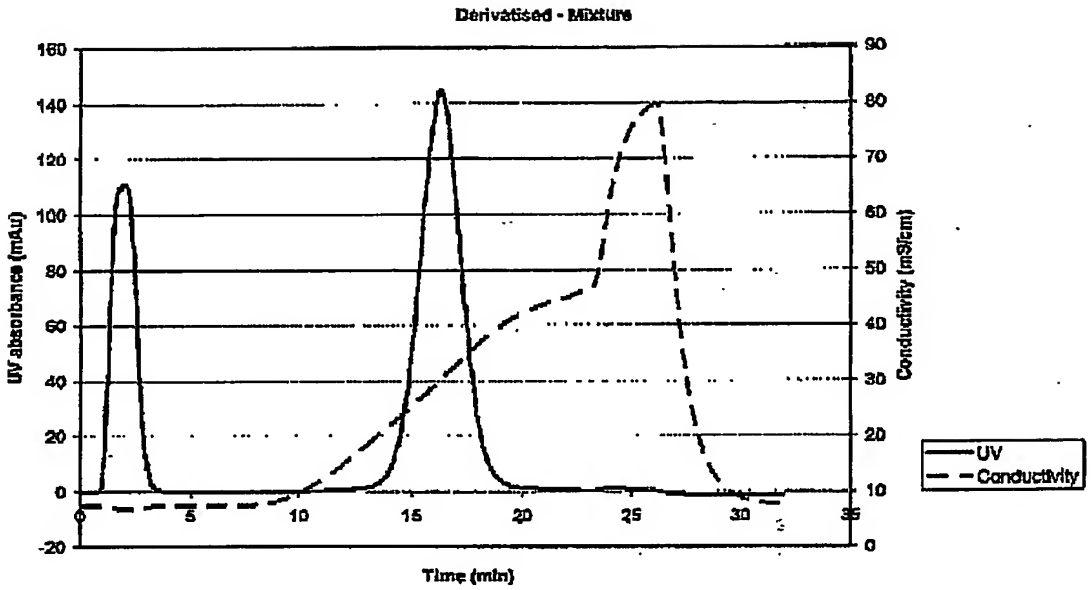

Derivatised - Lysozyme
UV absorbance (mAu)
Conductivity (mS/cm)
Time (min)
UV
Conductivity

Figure 6


Derivatised - Mixture
UV absorbance (mAu)
Conductivity (mS/cm)
Time (min)
UV
Conductivity